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Evaluation of the Regioselectivity of Human UDP-Glucuronosyltransferase Isozymes with Three Common Sub-Classes of Flavonoids Via Metal Complexation and Tandem Mass Spectrometry

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Evaluation of the Regioselectivity of Human UDP-Glucuronosyltransferase Isozymes with Three Common Sub-Classes of Flavonoids Via Metal Complexation and Tandem Mass Spectrometry

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Evaluation of the Regioselectivity of Human UDP-Glucuronosyltransferase Isozymes with Three Common Sub-Classes of Flavonoids Via Metal Complexation and Tandem Mass Spectrometry

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Based on reactions with two flavanones, three flavonols, and five flavones the regioselectivities of twelve human UDP-glucuronosyltransferase (UGT) isozymes were elucidated. The various flavonoid glucuronides were differentiated based on LC-MS/MS fragmentation patterns of [Co(II)(flavonoid – H)(4,7-diphenyl-1,10-phenanthroline)2]+ complexes generated upon post-column complexation. Glucuronide distributions were evaluated to allow a systematic assessment of the regioselectivity of each isozyme. The various UGT enzymes, including eight UGT1A and four UGT2B, displayed a remarkable range of selectivities, both in terms of the positions of glucuronidation and relative reactivity with flavanones, flavonols and flavones. The UGT1A enzyme selectivities are affected by the presence of a hydroxyl group at the 3, 6, 4', or 3' positions as well as by the presence of a methoxy at the 3' position. The UGT2B enzymes show poor to no reactivity with the flavonols or flavones. This result implies that the greater planarity of the flavonols and flavones compared to structure of flavanones inhibits interaction with the UGT2 enzymes. For baicalein and scutellarein, three of the UGT1A isozymes (1A8,

1A9, and 1A10) resulted in the formation of 6-O glucuronides, enabling the fragmentation rules for the metal complexation/MS/MS strategy to be expanded.

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Chapter 1: Regioselectivity of Human UDP-Glucuronosyltransferase Isozymes in Flavonoid Biotransformation by Metal Complexation and Tandem Mass Spectrometry

1.1 INTRODUCTION

Flavonoids, a class of polyphenols found in fruits and vegetables, have been shown not only to have anti-inflammatory properties but also to exhibit promising chemopreventive properties against cancer and cardiovascular disease.[1,2] The positive bioactivities of flavonoids have been demonstrated in a variety of *in vitro*, *in vivo*, and case control studies.[2–4] In recent years the presumed chemopreventive properties have been under closer scrutiny due to the poor bioavailability of most unmodified flavonoid aglycones in the body[5], coupled with the growing documentation that flavonoids undergo extensive biotransformation.[6–8] The metabolism of flavonoids has a great impact on their absorption and distribution, and importantly biotransformation can substantially alter the chemical properties of the flavonoids, such as altering the bioactivities.[9]

Most flavonoids are found naturally as glycosylated forms in fruits and vegetables. When ingested the flavonoid glycosides undergo deglycosylation by β-glucosidase or lactose phloridzin hydrolase enzymes primarily found in the small intestine.[10] After loss of their sugar side-chains, flavonoids are rapidly metabolized by mainly Phase II enzymes found in small intestine, kidneys, and most importantly the liver .[10] This process results in glucuronidation, sulfation, methylation, or hydroxylation depending on the nature of the interacting enzyme.[11] Any flavonoid compound metabolized or unmodified that is not absorbed prior to reaching the large intestine may

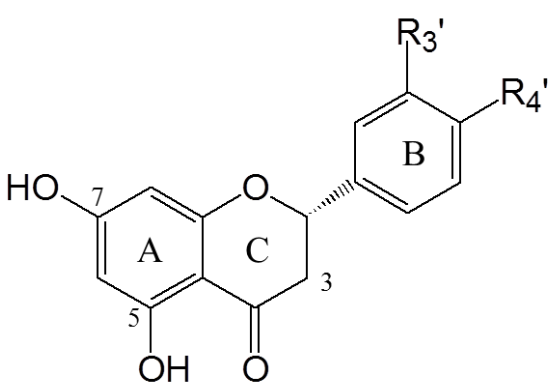
be absorbed by microflora, a process leading to decomposition of the flavonoid by ring fission and causing the release of small phenolic acids that are excreted in the urine.[10] Since most of the flavonoids ingested are conjugated and consequently absorbed as conjugates, there has been increasing interest in understanding the formation, uptake, distribution, and chemopreventive properties of the conjugates. To facilitate such investigations, the development of sensitive analytical methods to characterize, identify and track the flavonoid conjugates is paramount.

Glucuronidation of flavonoids is carried out in the body by the UDP-glucuronosyltransferase (UGT) family of enzymes. These enzymes have been found in every major organ involved in digestion, as well as the kidneys and liver.[11] To date, nineteen different isomers of the UGT enzyme have been identified[12], which are categorized into three different subgroups (UGT1As, UGT2As and UG2Bs). There are nine isoforms of the UGT1A group and seven in the UGT2B group, and together they play a major role in Phase II metabolism. The role of UGT2A isoforms remains unknown.[12] UGT enzymes catalyze the addition of glucuronic acid at a hydroxyl group, carboxylic acid, sulfide group, amine, or in rare cases a methyl group.[11] Flavonoids possessing one to multiple hydroxyl groups may undergo O-glucuronidation at various positions when metabolized by UGT enzymes. However, the specific positions which are glucuronidated by each enzyme are still not fully established.

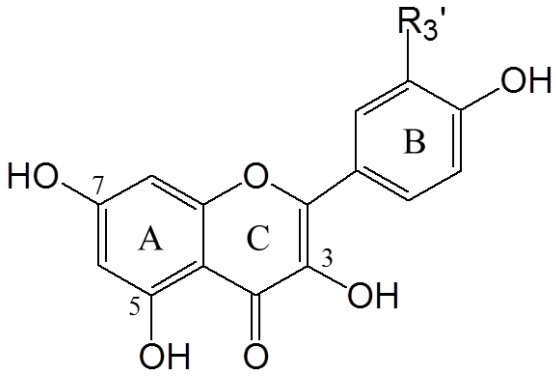
The structural characterization of flavonoids and their metabolites has proven to be a challenging task. All flavonoids share the same basic three-ring structure and may differ by the position of a single functional group, making their positive identification

difficult by many analytical methods. Mass spectrometry has proven to be one of the most effective tools for identification of flavonoids, in large part due to the informative fragmentation patterns generated by collision induced dissociation (CID) upon application of MS/MS strategies[13], especially when coupled with HPLC to allow separation of complex mixtures of flavonoids.[14–20] The Brodbelt lab has extended the capabilities of MS/MS methods for differentiation of flavonoids by formation of complexes containing a flavonoid, a metal, and an auxiliary organic ligand.[21] These complexes, upon CID, give unique fragmentation patterns that allow confident identification and differentiation of flavonoids, even for isomers. Brodbelt and coworkers have evaluated a number of metal complexation approaches and shown their versatility[22–29], including the adaptation of the methods for identification of metabolites in urine and plasma.[30–33] More recently, the metal complexation/MS/MS methodology has been applied to gain insight into the regioselectivity of the UGT1A1 enzyme with various flavonoids.[34] In this prior study, the products were confidently identified and the distributions of various glucuronidated products were quantified.

In this present study, I have expanded our investigation of the selectivity of glucuronidation of the twelve most common UGT enzymes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17) for five of the most commonly consumed flavonoids (hesperetin, isorhamnetin, kaempferol, naringenin, quercetin). (Figure 1.1) While biotransformation of flavonoids has been an area of much interest[12,35–40], this is the first time the isomeric flavonoid glucuronide products of such a large array of enzymatic syntheses have been determined, thus providing detailed insight into the selectivities of



Flavanones (MW)	R3’	R4’
Hesperetin (302)	OH	OCH_3
Naringenin (272)	H	OH



Flavonols (MW)	R3’
Isorhamnetin (316)	OCH_3
Kaempferol (286)	H
Quercetin (302)	OH

Figure. 1.1: Structures of flavonoids

the UGT isozymes. This systematic study provides benchmark data for assessment of UGT enzymatic regioselectivity and establishes predictive correlations of biotransformation upon consumption of flavonoids.

1.2 EXPERIMENTAL

1.2.1 Materials

All UDP-glucuronosyltransferase isozymes were purchased from BD Biosciences (Woburn, MA, USA). UDP-Glucuronic acid (UDPGA) trisodium salt, 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen), cobalt(II) bromide, hesperetin, naringenin, isorhamnetin, kaempferol, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, HPLC grade water, potassium phosphate, and methanol were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

1.2.2 Synthesis of Flavonoid Glucuronides by UGT Enzymes

The procedure for the glucuronidation reactions was modified from the protocol reported in Davis et al.[34] Each enzyme was divided into 25 μL aliquots and stored at -80 °C until use. The following reaction was set up for each combination of UGT enzyme (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17) and flavonoid (hesperetin, isorhamnetin, kaempferol, naringenin, quercetin). All volumes for this experiment were delivered appropriate micropipettes. The synthesis was carried out by adding 2 mM aqueous UDPGA (65 μL), 20 mM potassium phosphate buffer pH 7.0

(378 μL), and 10 mM methanolic solution of flavonoid (6.25 μL) to a microcentrifuge tube. The reaction was started by addition of 25 μL of a UGT enzyme (5 mg/mL). This concentration of enzyme was used based of experiments out lined by Plumb et al.[41] The mixture was incubated at 37 °C overnight. To stop the reaction, 1.5 mL of acetone was added. The tubes were centrifuged for 10 min at 16,000 g. The supernatant was removed and the acetone was evaporated by placing the tubes in a Savant DNA120 SpeedVac Concentrator (Thermo Electron, Waltham, MA, USA) on low heat for 1 hour and 40 min. The remaining mixture was refrigerated until analysis. It was brought to our attention that having flavonoids in methanol could lead to a decrease in enzyme activities. In a study by Easterbrook et al. the activities of UGT enzymes were evaluated in the presences of organic solvents at various concentrations, and they showed that there were no apparent effects to enzyme activities with a solution that has up to 2% methanol, so adding our flavonoids via a methanol solution does not have a great effect on the UGT enzymes ability to modify flavonoids.[42]

1.2.3 LC-MS/MS Analysis

LCMS analysis of the flavonoid glucuronides was undertaken using a Waters Alliance 2695 HPLC system (Milford, MA, USA) or a Hitachi L-7000 HPLC system (Hitachi High Technologies America, Pleasanton, CA, USA) and LCQ Duo quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) with electrospray ionization (ESI). The column was a Waters Symmetry C18 column, 2.1 x 50 mm, 3.5 μm particle size, with a guard column. The injection volume was 30 μL. The mobile phase

was 0.33% formic acid in water (A) and 0.33% formic acid in acetonitrile (B). The gradient used began at 15 % B and increased to 40 % over 30 min.

Samples were first analyzed in the negative ESI mode in order to search for flavonoid glucuronides. The spray voltage was set at 4.5 kV, the heated capillary temperature was 200 °C, and the automatic gain control was set to 5 x 10^7 ions with a maximum injection time of 500 ms and 5 microscans averaging. All other parameters were set to obtain optimal signal. The positive ESI mode was used for MS/MS analysis of the flavonoid/metal complexes. The metal complexes were formed by post-column addition of a methanolic solution of 5 μM $CoBr_2$ and 4,7-dpphen, which was infused at a rate of 20 μL/min controlled by a syringe pump. The spray voltage for the positive ion mode was set to 5 kV, and the heated capillary temperature was 200 °C. The automatic gain control for MS/MS was set to 2 x 10^7 ions with a maximum injection time of 500 ms and 5 microscan averaging, the isolation width was set to 4 Da, and a collision energy of 35% normalized collision energy was used for collision induced dissociation.

1.2.4 LC-UV Monitoring of Reaction Products

In order to estimate the relative product distributions of different flavonoid glucuronides for each enzymatic reaction leading to multiple products, the peak area for each resulting product was integrated based on its LC-UV chromatographic profile at 360 nm. The area of each product peak was divided by the total area of all product peaks in order to calculate the product distributions as percentages. This percentage was rounded to the nearest 5%.

1.2 RESULTS

Our objective was to map the formation of various flavonoid glucuronides for each glucuronosyltransferase enzyme. In order to differentiate the flavonoid glucuronide isomers, MS/MS spectra of the metal complexes $[Co(II)\ (FG - H)\ (4,7\text{-dpphen})_2]^+$ were analyzed along with HPLC retention times (where FG represents a flavonoid glucuronide). These metal complexes were produced via post-column complexation in LCMS runs of the product mixtures obtained for each flavonoid/glucuronosyltransferase combination. Whereas the fragmentation patterns of deprotonated flavonoid glucuronides are typically not distinctive for isomers, the MS/MS spectra of the metal complexes allows differentiation and confident elucidation of the isomers. As described previously[30,34], the metal complexes containing 7-O-glucuronides show losses of the auxiliary ligand (-Aux) and the glucuronic acid moiety (-GlcA), both individually or together (–(GlcA + Aux)), upon CID. The loss of the flavonoid aglycon (-Agl) is also a characteristic fragment of 7-O-glucuronides. The metal complexes of the 5-O-glucuronides and 3-O-glucuronides both show a prominent loss of the glucuronic acid moiety in conjunction with the auxiliary ligand (–(GlcA + Aux)); moreover, the 5-O product has been shown to elute prior to the 7-O product[34] whereas the 3-O-glucuronide elutes after. For B-ring-glucuronides, the characteristic fragments include the loss of the auxiliary ligand (–Aux) and the losses of both the auxiliary ligand and glucuronide moiety together (–(GlcA + Aux)).[30] 4'-O-glucuronides elute before 3'-O glucuronides

and after the 3-O-glucuronides. These systematic MS/MS and elution patterns were utilized for assignment of the glucuronide products in the present study. (A table listing all the fragments found for the products observed in this paper in Table 1.1)

Once the enzymatic reactions were quenched, each of the incubates was centrifuged and the supernatant was screened by LCMS in the negative ESI mode. The resulting total ion chromatograms were searched both for the unreacted flavonoid aglycon, monoglucuronidated flavonoid products (aglycon + 176), and diglucuronidated products (aglycon +176 +176) based on the m/z values of the anticipated products. A typical total ion chromatogram obtained for the hesperetin product mixture is shown in Figure 1.2a with extracted ion chromatograms shown in Figures 1.2b and 1.2c for deprotonated hesperetin (m/z 301) and the monoglucuronidated products (m/z 477). No diglucuronidated products were detected. After screening the enzymatic incubates, then the metal complexes were generated upon LCMS by post-column complexation and subjected to CID for structural characterization. Examples of the resulting MS/MS spectra are shown in Figure 1.3 for hesperetin glucuronides produced from UGT1A6 and in Figure 1.4 for kaempferol glucuronides produced from UGT1A1. All products identified are summarized for the two classes of flavonoids (flavanones and flavanols) in Table 1.2 along with the distribution of products based on integration of the chromatographic peak areas of each product and unreacted flavonoid.

Table 1.1: Observed Fragments For All UGT Products

Hesperetin	Major Fragments Observed	Minor Fragments Observed
5-O-Glucuronide	-(GlcA + Aux)	-GlcA
7-O-Glucuronide	-(GlcA + Aux); -GlcA; -Aux	-(Aux + Alg)**;** -Agl
3'-O-Glucuronide	-(GlcA + Aux)	-(Aux + Agl)**;** -Aux;-Agl; -GlcA
Naringenin		
5-O-Glucuronide	-(GlcA + Aux)	-GlcA
7-O-Glucuronide	-(GlcA + Aux); -GlcA; -Aux	-(Aux + Alg)**;** -Agl
Isorhamnetin		
5-O-Glucuronide	-(GlcA + Aux)	-GlcA; -Aux
7-O-Glucuronide	-(GlcA + Aux); -GlcA; -Aux	-Agl
3-O-Glucuronide or 4'-O-Glucuronide	-(GlcA + Aux)	-GlcA; -Aux
Kaempferol		
5-O-Glucuronide	-(GlcA + Aux)	-GlcA
7-O-Glucuronide	-(GlcA + Aux); -Aux	-GlcA; -(Aux + Alg); -Agl
4'-O-Glucuronide	-Aux	-(Aux + Agl); -(GlcA + Aux); -GlcA
Quercetin		
5-O-Glucuronide	-(GlcA + Aux)	-
7-O-Glucuronide	-(GlcA + Aux); -Aux	-GlcA; -(Aux + Alg); -Agl
4'-O-Glucuronide	-(GlcA + Aux); -Aux	-GlcA
3'-O-Glucuronide	-(GlcA + Aux); -Aux	-GlcA

Note: –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon) Fragments that were above 50% abundance are listed as major fragments. Fragments that were below 50% abundance are listed as minor fragments.

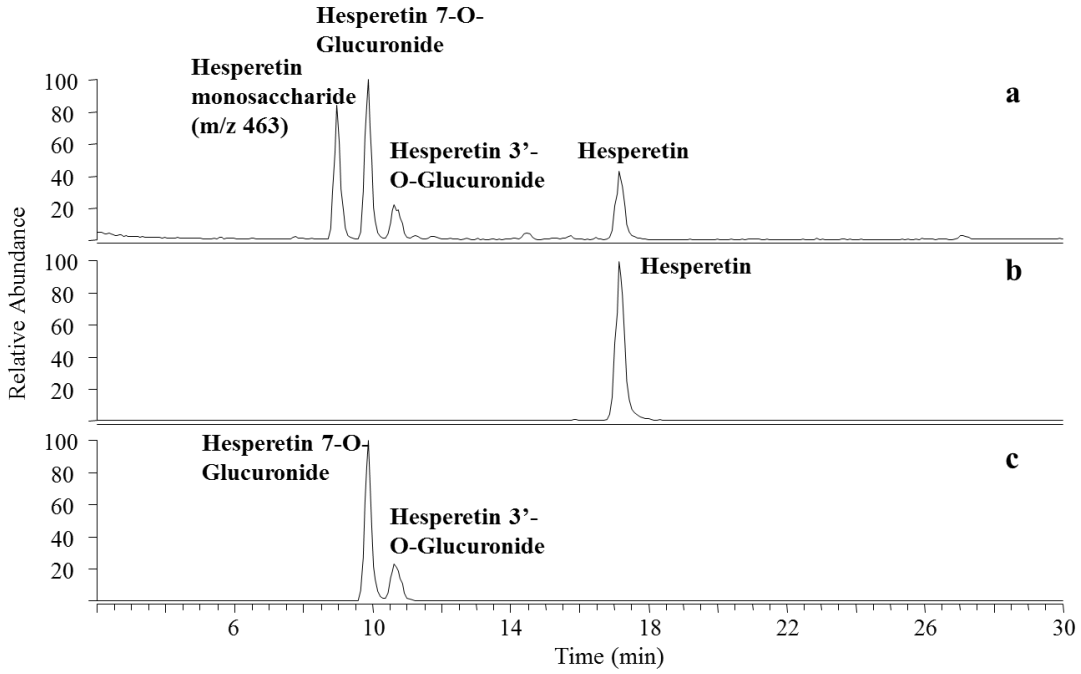


Figure. 1.2: a) Total ion chromatogram for UGT1A10 reaction with hesperetin, including background subtraction. b) Selected ion chromatogram for deprotonated hesperetin (m/z 301) . c) Selected ion chromatogram for deprotonated hesperetin monoglucuronide (m/z 477).

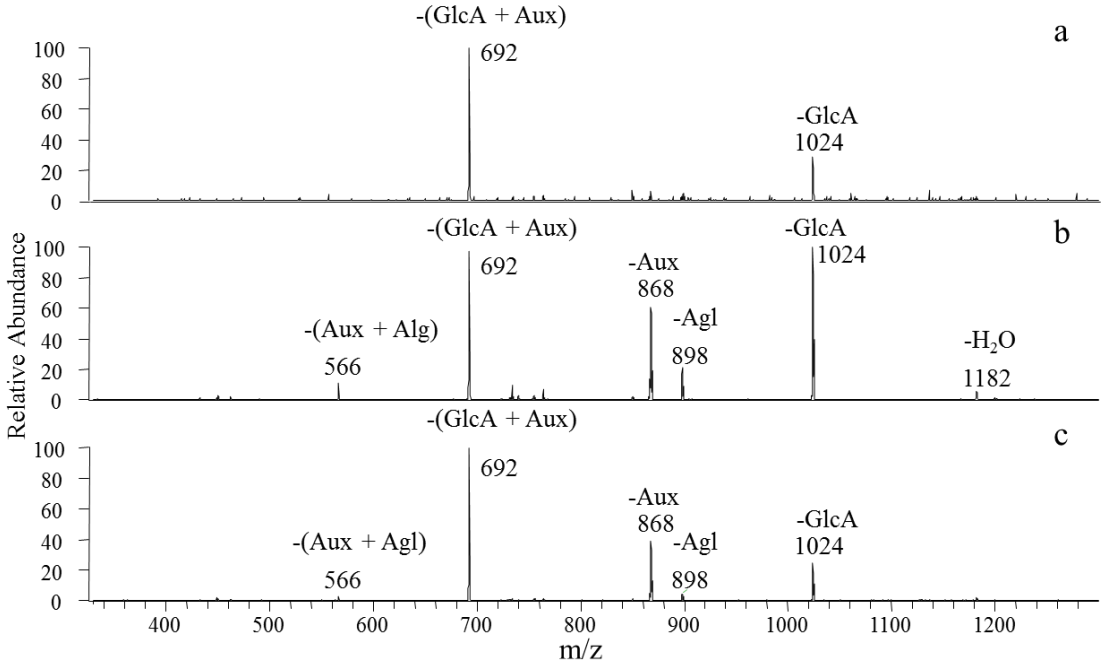


Figure 1.3: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A6/hesperetin products: a) 5-O glucuronide, m/z 1200 b) 7-O glucuronide, m/z 1200 c) 3'O- glucuronide, m/z 1200. –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon)

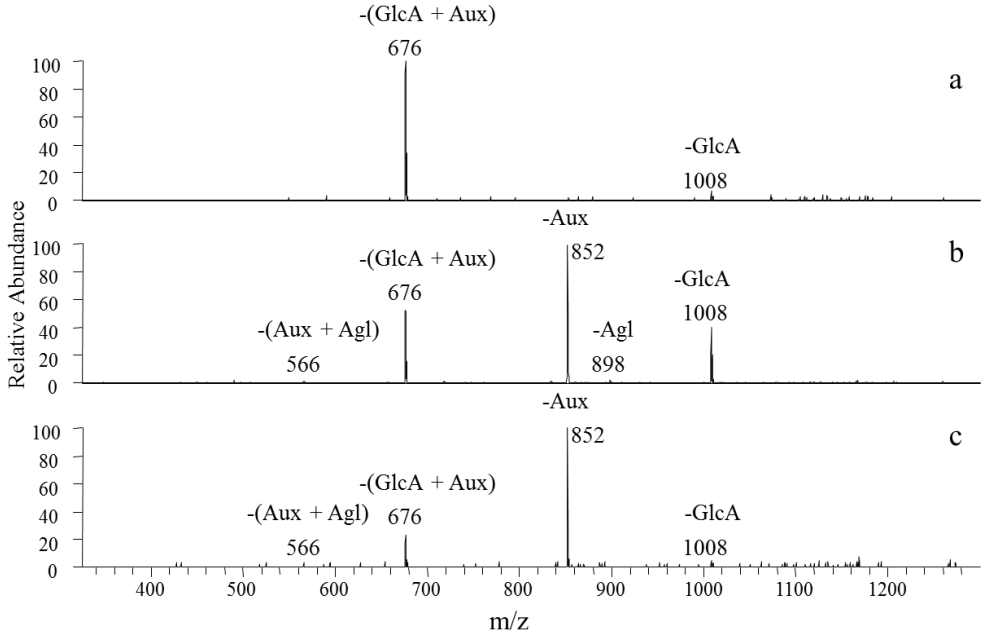


Figure 1.4: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A1 kaempferol products: a) 5-O-Glucuronide, (m/z 1184) b) 7-O-Glucuronide, (m/z 1184) c) 4'-O-Glucuronide, (m/z 1184). –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon)

Table 1.2: Glucuronide product distributions relative to unreacted flavonoid

Hesperetin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
Hesperetin	60	15	100	10	45	70	30	75	80	60	100	100
5-O-Glucuronide	-	-	-	Trace	-	-	-	-	-	-	-	-
7-O-Glucuronide	30	10	-	90	5	10	30	15	20	30	Trace	Trace
3'-O-Glucuronide	10	75	-	Trace	50	20	40	10	Trace	10	Trace	Trace
Naringenin	**1A1**	**1A3**	**1A4**	**1A6**	**1A7**	**1A8**	**1A9**	**1A10**	**2B4**	**2B7**	**2B15**	**2B17**
Naringenin	40	50	100	30	80	65	20	90	70	80	95	95
5-O-Glucuronide	-	-	-	Trace	-	-	-	-	-	-	-	-
7-O-Glucuronide	50	50	-	70	20	35	80	10	30	20	5	5
4'-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
Isorhamnetin	**1A1**	**1A3**	**1A4**	**1A6**	**1A7**	**1A8**	**1A9**	**1A10**	**2B4**	**2B7**	**2B15**	**2B17**
Isorhamnetin	80	35	100	100	90	100	95	95	100	100	100	100
5-O-Glucuronide	-	-	-	-	10	-	5	Trace	-	-	-	-
7-O-Glucuronide	-	50	-	-	Trace	-	-	Trace	-	-	-	-
3-O-Glucuronide	(20)	(15)	-	-	-	-	-	(5)	-	-	-	-
4'-O-Glucuronide	(20)	(15)	-	-	-	-	-	(5)	-	-	-	-
Kaempferol	**1A1**	**1A3**	**1A4**	**1A6**	**1A7**	**1A8**	**1A9**	**1A10**	**2B4**	**2B7**	**2B15**	**2B17**
Kaempferol	40	90	100	60	75	90	75	100	95	100	100	100
5-O-Glucuronide	5	Trace	-	30	10	Trace	10	Trace	-	Trace	-	-
7-O-Glucuronide	50	10	-	10	15	5	15	Trace	5	Trace	-	-
3-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
4'-O-Glucuronide	5	Trace	-	Trace	-	5	Trace	Trace	-	Trace	-	-
Quercetin	**1A1**	**1A3**	**1A4**	**1A6**	**1A7**	**1A8**	**1A9**	**1A10**	**2B4**	**2B7**	**2B15**	**2B17**
Quercetin	25	55	100	95	100	100	70	95	100	90	100	100
5-O-Glucuronide	-	-	-	Trace	-	-	-	-	-	Trace	-	-
7-O-Glucuronide	5	25	-	5	-	Trace	30	5	-	-	-	-
3-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
4'-O-Glucuronide	10	5	-	-	-	Trace	-	Trace	-	-	-	-
3'-O-Glucuronide	60	15	-	-	-	Trace	-	Trace	-	10	-	-

All values are percentages of total product distribution. Values in parenthesis indicate products with unconfirmed structures, meaning that two alternatives are possible. A dash is used to indicate the absence of a product. All values rounded to the nearest 5%. The average standard deviation is $\pm$5%.

1.3.1 Flavanone Glucuronides

Hesperetin formed monoglucuronidated products upon exposure to all the glucuronosyltransferases except for UGT1A4 (which in fact produced no glucuronides for any of the flavonoids). These products were analyzed by the metal complexation/MS/MS method described in the Experimental section and assigned based on their characteristic fragmentation patterns and retention times. For UGT1A1, 1A3, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17, two products were found (Table 1.2). The MS/MS spectrum of the first glucuronide product showed losses of GlcA, Agl, and Aux, which is characteristic of 7-O-glucuronide products. The second eluting product yielded the prominent loss of (GlcA + Aux) as well as the loss of Aux, which as described above is characteristic of 3'-O-glucuronidation. For UGT1A6, hesperetin showed a total of three products (see corresponding MS/MS patterns in Figure 1.3). Two of the products are identical to those described above for the other glucuronosyltransferase reactions. The third product eluted prior to the 7-O-glucuronide, and the MS/MS spectrum shows one prominent loss of (GlcA + Aux), thus confirming this product as a 5-O-glucuronide.

For naringenin all of the glucuronosyltransferases resulted in only a single product except for UGT1A4, which promoted no products at all and UGT1A6 which led to the formation of two products. The single dominant product dissociated by pathways characteristic of a 7-O-glucuronide (losses of GlcA, Agl, and Aux). For the UGT1A6

reaction the second product showed a prominent loss of (GlcA + Aux) which is assigned as the 5-O-glucuronide owing to its elution prior to the 7-O-glucuronide product. (See CID mass spectra in Figure 1.5.)

1.3.2 Flavonol Glucuronides

Upon reactions with most of the glucuronosyltransferases, isorhamnetin displayed no reactivity in the presence of the UGT1A4, 1A6, 1A8, 2B4, 2B7, 2B15, and 2B17 enzymes. Reaction with the UGT1A3 enzymes led to the formation of two products. The first product showed losses of GlucA, Agl and Aux upon CID, allowing ready assignment as the 7-O-glucuronide. The second product demonstrated loss of (GlcA + Aux) as the major product, and also showed a loss of Aux though this fragment occurred in very low abundance. This product could either be a 3-O glucuronide or a 4'-O glucuronide because its elution after the 7-O glucuronide ruled it out as a 5-O glucuronide. Moreover the 3' position has a methoxy group, and the absence of a methyl group loss allowed a 3'-glucuronide structure to be ruled out. The minor loss of Aux in the MS/MS spectrum suggested that this was the 4'-O glucuronide, but the evidence was somewhat inconclusive due to the lack of other diagnostic ions.

The UGT1A1 enzyme promoted the formation of one product for isorhamnetin that dissociated by a major loss of (GlcA + Aux) and a minor loss of Aux which had a very low abundance. Since there are no other products in this reaction, confident identification of this product was not possible; however comparing the elution time of this product with confirmed products of other isorhamnetin/glucuronidation reactions

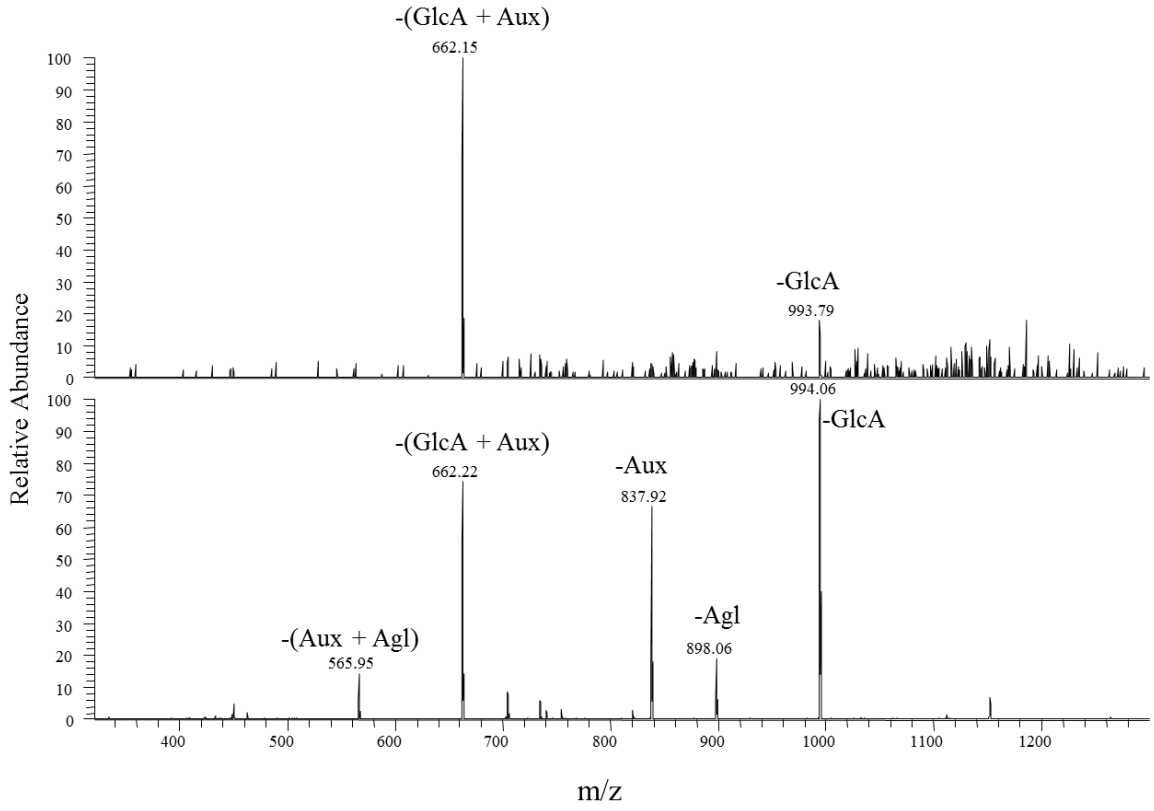


Figure 1.5: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all naringenin products: a) 5-O-Glucuronide, (m/z 1170) b) 7-O-Glucuronide, (m/z 1170). –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon)

shows that this product has a retention time matching that of the second product of the UGT1A3, which suggest that this product also having the minor loss of Aux in the CID spectrum appears to be a 4'-O-glucuronide. However this product could not be confidently distinguished between the 3-O-glucuronide and 4'-O-glucuronide structures. The UGT1A7 enzyme resulted in formation of two products upon reaction with isorhamnetin. The first product exhibited the dominant loss of (GlucA + Aux), and the second product displayed all the characteristic losses of a 7-O-glucuronide species (-GlucA, -Agl, -Aux). The first product corresponded to the 5-O-glucuronide due to its elution prior to the 7-O product. The UGT1A9 enzyme promoted formation of one product that dissociated by loss of (GlcA + Aux) upon CID, indicating that this could be a 3-O or 5-O glucuronide. With only a single product eluting, the only way this product can be identified is based on comparison of retention times to products from those of the 1A7 reaction. The elution time of the UGT1A9 product matched that of the 5-O-glucuronide identified from the 1A7 reaction mixture, thus confirming the UGT1A9 product as 5-O-glucuronide. (See CID mass spectra in Figure 1.6).

The reaction of isorhamnetin with UGT1A10 yielded three products. The first two eluting products were identified as the 5-O glucuronide and 7-O glucuronide respectively; this is because the second product showed losses of GlucA, Agl, and Aux upon CID, allowing it to be identified as the 7-O glucuronide. The first eluting product showed a dominant loss of (GlucA + Aux), which because it eluted before the 7-O glucuronide can be readily assigned as the 5-O glucuronide. The third product showed a loss of (GlcA + Aux) as the major product, and also exhibited a loss of Aux although this

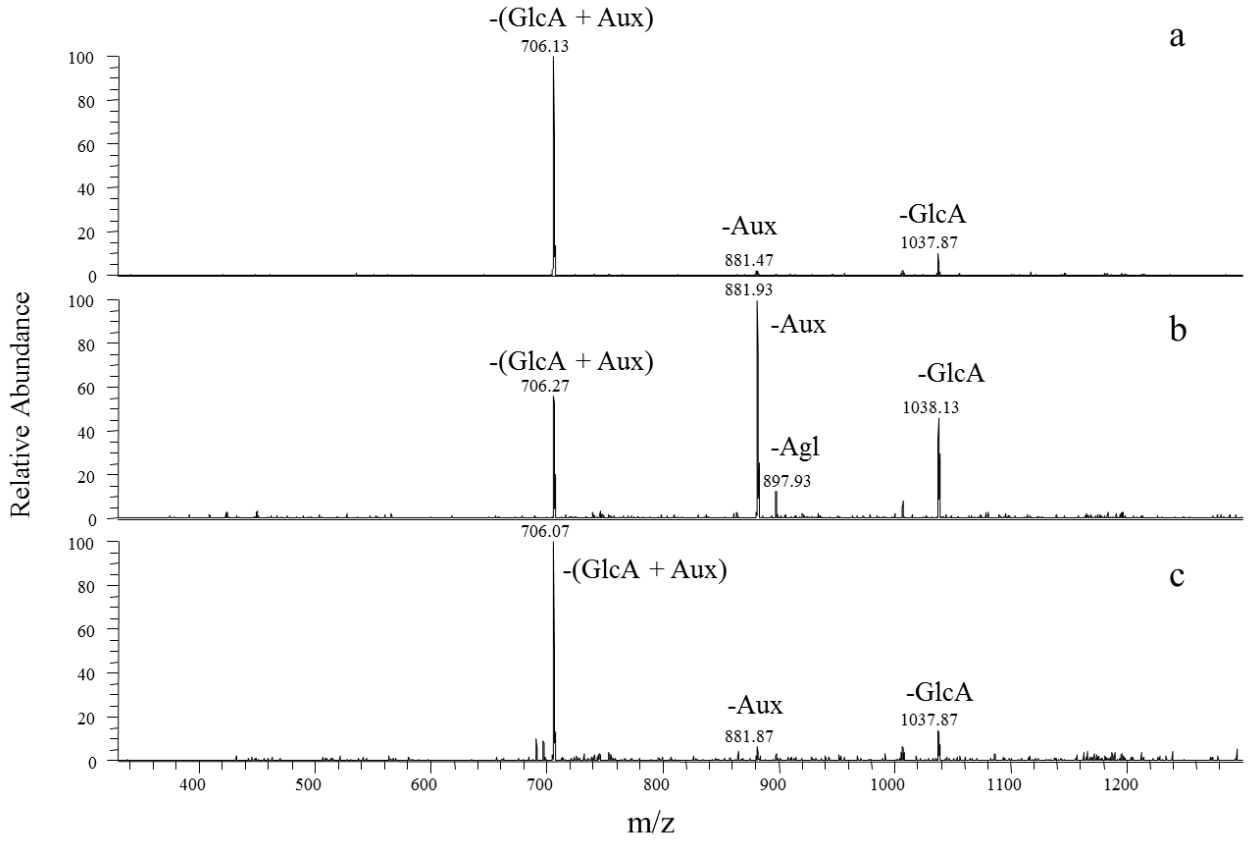


Figure 1.6: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all isohamnetin products: a) 5-O-Glucuronide, (m/z 1214) b) 7-O-Glucuronide, (m/z 1214) c) 4'-O-Glucuronide or 3-O-Glucuronide, (m/z 1214). –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon)

latter fragment occurred in very low abundance similar to that of the second product of the UGT1A3 reaction. This product could not be confidently distinguished between the 3-O glucuronide and 4'-O glucuronide structures. One possible way to identify this product would be if LC-NMR was used. In order to do this, however, a much larger quantity of the glucuronide products would be needed in order to perform a LC-NMR.

Kaempferol formed three glucuronides (5-O, 7-O, and 4'-O) with the exception of reactions promoted by UGT1A4, UGT2B15, and UGT2B17 which resulted in no products, UGT1A7 which yielded only two products, and UGT2B4 which led to only one glucuronide. As described above, the identities of the products were assigned based on their MS/MS patterns, as illustrated in Figure 4, and their relative retention times. Upon analysis of the UGT1A1 glucuronides, the first eluting product dissociated by losses of GlcA and (GlucA + Aux), the second product demonstrated losses of GlcA, Agl, and Aux, some occurring together, and the third product showed prominent losses of Aux and (GlcA + Aux). The first product was readily identified as kaempferol-5-O-glucuronide because of the characteristic sole major loss of (GlucA + Aux), as well as the fact that this product eluted prior to the second product, the latter which is confidently assigned as the 7-O-glucuronide based on its characteristic fragmentation pattern. The third product could conceivably be a 3-O or 4'-O glucuronide, both which should elute after the 7-O-glucuronide. However, the fact that the loss of Aux was observed along with the combined loss of (GlcA + Aux) ruled out the 3-O-glucuronide. For the reactions with the UGT1A7 enzyme, the two products formed included the 5-O-glucuronide and 7-O-glucuronide but not the 4'-O-glucuronide. The one product formed for the UGT2B4

reactions was identified as the 7-O- glucuronide product, while neither the 5-O-glucuronide nor the 4'-O- glucuronide were produced.

Quercetin has five possible glucuronidation sites, and it generated three different products upon reaction with the UGT1A1, 1A3, 1A8, and 1A10 glucuronosyltransferases. Upon CID, the first eluting product showed fragment pathways characteristic of 7-O-glucuronide, including losses of GlcA, Agl, and Aux. The next two products both showed losses of Aux and Aux + GlcA. The first of these two products is assigned as the 4'-O-glucuronide based on its elution prior to the other 3'-O-glucuronide. Reaction with the UGT1A9 enzyme resulted in only one product, the 7-O-glucuronide. The UGT1A6 enzyme predominantly led to formation of the 7-O-glucuronide as well as the 5-O product which was not observed for any of the enzymatic reactions of quercetin. This 5-O product was confirmed because it showed only one characteristic loss (-(GlcA + Aux)) and eluted prior to the 7-O-glucuronide. The UGT2B7 enzyme led to formation of two products, the first of which showed the loss of (GlcA + Aux) as the only major fragmentation pathway while the second product showed losses of Aux and (GlcA + Aux). The fragmentation pattern of this second product was consistent with either a 3'-O glucuronide or a 4'-O glucuronide. The similar MS/MS patterns and elution times of 3'-O and 4'-O glucuronides did not allow conclusive assignment of this particular quercetin product, however by performing a coelution of the products from the UGT1A3 reaction and products from this UGT2B7 reaction, allowed for 4'- glucuronide to be ruled out and the product to identified as a 3'-O modification. The first product was identified as a 5-O

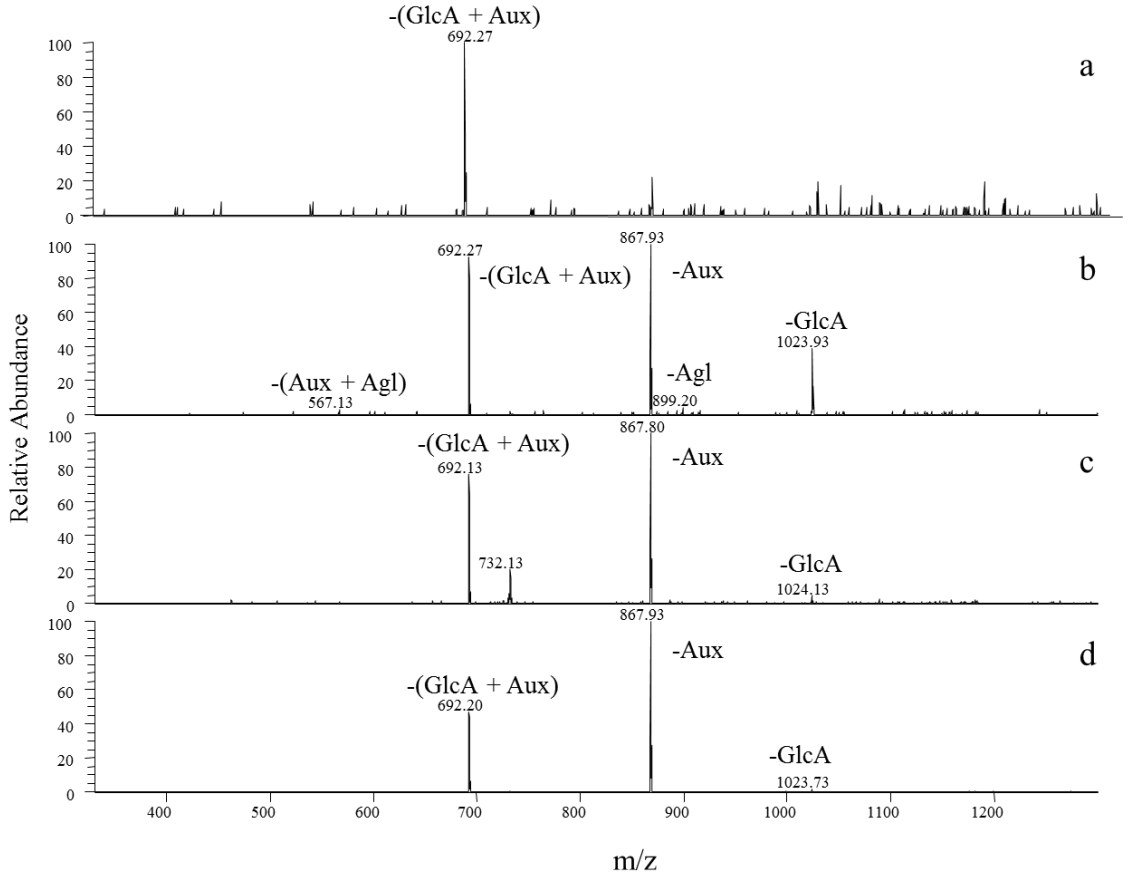


Figure 1.7: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all quercetin products: a) 5-O-Glucuronide, (m/z 1200) b) 7-O-Glucuronide, (m/z 1200) c) 4'-O-Glucuronide, (m/z 1200), d) 3'-O-Glucuronide (m/z 1200). –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon)

glucuronide because of its similar retention time to the 5-O product confirmed from the UGT1A6 reactions. (See CID mass spectra in Figure 1.7).

1.3.3 Selectivity Trends

In a recent study by Singh et al. the interaction of kaempferol with UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7 was evaluated.[40] In comparing the results for kaempferol found in their study with the ones reported here, there is on glaring difference that is noticed. That difference is the fact that in the study by Singh et al. modification at the 3-O position is observed for several of the enzymes, and in conjunction with that the results show a much lower likely hood that a 5-O product is formed. The only major difference between the two studies is the Singh and colleges used a UV method as the main method of analysis. It is unclear as to what could cause this difference to occur, but more data would have to be seen from the Singh et al. study to before making any firm conclusions.

Each UGT enzyme exhibited selectivity with respect to the sites of glucuronidation of flavonoids. To establish a benchmark for evaluating the array of UGT glucuronosyltransferases, the glucuronidation trends for 1A1 in the present study were first compared to a previous limited set of results obtained using the same enzyme and LCMS/MS analysis.[34] It was previously reported that UGT1A1 selectively modifies only the hydroxyl group at the 7 position, unless there is a hydroxyl at the 3' position in which

case UGT1A1 also modifies hydroxyl groups on the B ring.[34] The relative quantities of the hesperetin and quercetin glucuronidation products in this current study were in good agreement with those found in the previous study. The sole glucuronidation of naringenin at the 7-O position noted in the past study also matches the same finding in the present study. The glucuronidation of kaempferol, however, shows discrepancy based on our present results. Only the 7-O-glucuronide product was reported previously, unlike the three distinctive products (5-O, 7-O, and 4'-O) found in the present study. Isorhamnetin is the only flavonoid whose glucuronidation in the presence of UGT1A1 has not been reported previously, and it in fact yields just one product.

The results for the UTG1A3 enzyme show that its selectivity differs slightly from that of UGT1A1, with greater preference for glucuronidation of the 7-O position of the flavonols. The best example of this is reflected by the quercetin and isorhamnetin results, in which the production of 7-O glucuronides is enhanced for the UGT1A3 reactions. This is particularly evident in the reactions of isorhamnetin with UGT1A3 for which the 7-O-glucuronide product was by far the most abundant, a product not even detected upon reaction in the presence of UGT1A1. The selectivity of the UGT1A3 enzyme for hesperetin is likewise very different from that of the UGT1A1 reaction, in that the 3'-O glucuronide is the most abundant product, not the 7-O product. A previous recent study reported the glucuronidation of isorhamnetin, kaempferol, and quercetin with UGT1A3 and UGT1A9[35], but specific product identities were not assigned. In this past study three products were found for isorhamnetin, two for kaempferol, and four for quercetin.[35] For each of isorhamnetin and quercetin, this represents one additional product than reported

in the present study but for kaempferol it is one less, in regards to UGT1A3. Due to the lack of product identities, further comparisons to the present results were not possible.

Several other notable features emerged upon inspection of the glucuronidation results for the other enzymes. UGT1A6 proved to be unusual in that it is the only glucuronidase that is able to promote 5-O-glucuronidation of the two flavanones, hesperetin and naringenin, and also the flavonol quercetin. UGT1A7 and UGT1A9 exhibited a preference for modifying the 3' position of the hesperetin, but favored the 7-O position of naringenin and the flavonols. In particular, the 3'-O glucuronides were most abundant for hesperetin, whereas kaempferol and quercetin showed no or low abundance B-ring products. Instead the most abundant products for kaempferol were the 7-O and 5-O (A-ring) glucuronides. The UGT1A8 enzyme displayed a high preference for modification of flavonoids on the B-ring. For all the flavonoids that yielded multiple products, the most abundant glucuronide involved the B-ring. UGT1A10 preferentially glucuronidated the 7-O position, except for isorhamnetin which showed the 3-O or 4'-O position as the more highly favored sites for glucuronidation

The UGT2B enzymes (UGT2B4, UGT2B7, UGT2B15, and UGT2B17) all shared one similar characteristic: They all showed poor to no reactivity with the flavonols, suggesting that the general flavanol structure restricted interaction with the UGT2B enzymes or otherwise deactivated the glucuronidation reactions.

There are also notable differences in the glucuronidation selectivities based on subtle structural differences of the flavonoids. For example, naringenin and kaempferol are analogs, with the former being a flavanone and the latter being a flavanol with its

double bond on the C ring and hydroxyl at the 3-O position. The flavanol structure enhances the ability of the UGT enzymes to modify the 4'-O position. In no cases does naringenin undergo glucuronidation at the 4'-O position, whereas 4'-O glucuronide products are produced in nearly every reaction for kaempferol.

Kaempferol and quercetin also share similar structures except for the additional hydroxyl group at the 3' position of quercetin. The 3'-OH group proved to be a very reactive site for glucuronidation for quercetin (as well as the flavanone hesperetin). Another point of interest when comparing kaempferol and quercetin is that the presence of the hydroxyl at the 3' position seems to render the 5-O position almost completely inactive, except for in the cases of the UGT1A6 and the UGT2B7 enzyme, which both lead to 5-O glucuronide as a minor product.

Quercetin also is similar in structure to isorhamnetin with the only difference being that the 3'-O position is methylated for isorhamnetin. The methoxy at the 3' position appears to deactivate glucuronidation in general by the UGT enzymes since products are only detected for five of the UGT enzymes (UGT1A1, UGT1A3, UGT1A7, UGT1A9, and UGT1A10). Interestingly, isorhamnetin exhibited greater reactivity with UGT1A7 than was observed for quercetin (no products).

1.4 CONCLUSION

The regioselectivity of the reactions of twelve human UDP-glucuronosyl-transferase (UGT) isozymes with five common flavonoids were evaluated by LC-MS/MS with post-column metal complexation. Metal complexation results in the formation of

[Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ ions which are key for confident identification of the modification site promoted by a given UGT isozyme due to the more diagnostic fragmentation patterns than produced by conventional deprotonated flavonoid glucuronides. The UGT1A enzyme selectivities are affected by the presence of a hydroxyl group at the 3, 4', or 3' positions as well as by the presence of a methoxy at the 3' position. The UGT2B enzymes all share one similar trend: They all show poor to no reactivity with the flavonols. This result implies that the greater planarity of the flavonols compared to structure of flavanones or the additional hydroxyl group at the 3 position inhibits interaction with the UGT2 enzymes. This study also shows the effectiveness of metal complexation/tandem mass spectrometry in conjunction with HPLC retention times for identification of flavonoid monoglucuonides. Of the 60 reactions reported in this study only three resulted in a product that could not be differentiated.

Chapter 2:Flavone Glucuronide Isomers Identified by Metal Complexation and Tandem Mass Spectrometry: Regioselectivity of UDP-Glucronosyltransferase Isozymes in the Biotransformation of Flavones

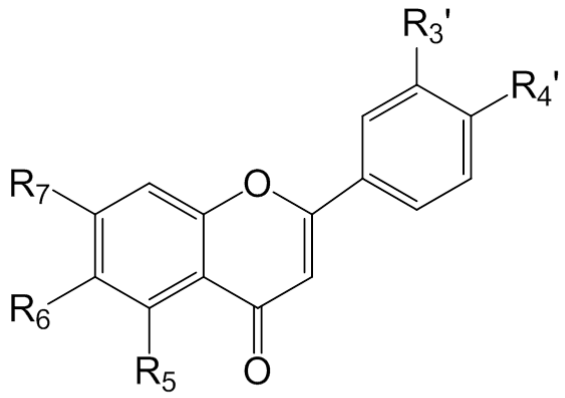
2.1 INTRODUCTION

The biotransformation of flavonoids has been a topic of increasing research activity over the past decade due to the interest in mapping the correlation between the beneficial chemopreventive properties of flavonoids and the structures of the active compounds in the body.[1,2] Moreover, understanding the bioavailabilities of flavonoids demands consideration of the metabolism of native flavonoids upon consumption.[3,6,7] In this context, there have been a number of strategies aimed at elucidating the structures of the biotransformation products of flavonoids, a task that proves challenging due to the number of ways that flavonoids can be metabolized and the number of isomeric structures that may defy facile differentiation.[43] Although flavonoids are typically glycosylated in fruits and vegetables, they are readily enzymatically deglycosylated by β-glucosidases or lactose phloridzin hydrolases in the small intestine after ingestion.[10] Once the sugar side-change is removed, flavonoids are most frequently modified by addition of a glucuronic acid or sulfate group or in some cases by methyl or hydroxyl groups.[10,11] These processes are mainly carried out by Phase II enzymes found in the small intestine, kidneys, and the liver.[11] It is these conjugated flavonoid species that are absorbed by the body. In fact, it has been shown that flavonoid aglycones in general have poor bioavailability, a factor which has motivated many investigations of metabolism in order

to rationalize how inactive compounds or ones with poor bioavailability may exert positive health benefits.[43]

One of the most common conjugates formed during metabolism are O-glucuronides. Glucuronidation arises from the UDP-glucuronosyltransferase (UGT) family of enzymes.[11] This family is split into three main sub groups and contains a total of nineteen different isoforms including nine UGT1As, three UGT2As, and seven UGT2Bs. Currently it is known that the UGT1A group and UGT2B group play a major role in Phase II metabolism; however little is known about the function of the UGT2A group.[12] With respect to their biotransformative role, UGT enzymes catalyze the addition of glucuronic acid to any hydroxyl group, resulting in formation of O-glucuronide products.[11] This rather ubiquitous glucuronidation process makes it particularly difficult to identify the products with confidence as flavonoids may have multiple hydroxyl groups. Reports have shown that the addition of glucuronic acid to a flavone can greatly alter the bioactivity of flavones, thus causing stimulating efforts to unravel the formation and distribution of the glucuronides as well as the effects of glucuronidation on the bioactivities of the flavonoids. [5,8]

Flavones, a sub-class of flavonoids, are distinguished from other sub-groups of flavonoids by a double bound between the 2 and 3 positions on the C ring and a lack of a hydroxyl group at the 3 position. The basic structure is shown in Figure 2.1. Flavones are found in various types of fruits and vegetables, as well many different herbs.[44] This sub-class of flavonoids has been reported to very biologically active and may play a role in countering diabetes mellitus, arteriosclerotic vascular disease and even breast cancer. [45,46]



Flavones (MW)	R5	R6	R7	R3'	R4'
Chrysin (254)	OH	H	OH	H	H
Apigenin (270)	OH	H	OH	H	OH
Luteolin (286)	OH	H	OH	OH	OH
Baicalein (270)	OH	OH	OH	H	H
Scutellarein (286)	OH	OH	OH	H	OH
5-Hydroxyflavone (238)	OH	H	H	H	H
6-Hydroxyflavone (238)	H	OH	H	H	H
7-Hydroxyflavone (238)	H	H	OH	H	H
6,7-Dihydroxyflavone (238)	H	OH	OH	H	H

Figure 2.1: Structures of flavones

Numerous studies have demonstrated these positive chemopreventive properties in a variety of *in vitro, in vivo,* and case control studies.[37,47,48] As alluded to above, structural characterization of flavone-O-glucuronides is difficult. With UGT enzymes able to modify any hydroxyl group, the formation of different isomers is feasible. For many years identification of these glucuronides isomers has been performed by the comparison of retention times with synthesized standards of the different isomers; however this method is limited by the lack of availability of standards and the complexity of synthesizing and purifying such compounds.[35,37–39,41,49–51] Recently, methods have been developed to facilitate differentiation of these types of isomers based on advanced chromatographic methods with tandem mass spectrometry and/or use of the UV spectral shift method to assign conjugation positions.[30,52,53] Of these methods tandem mass spectrometry of flavonoid/metal complexes has proven to be extremely effective.[21,23,27] For example, we have shown that using metal complexation is an effective method for differentiation of isomeric flavonoids and their glucuronides, including ones in the sub-class of flavones.[22,24–26,28,29,31,32] This metal complexation strategy has also been adapted for the identification of flavonoid glucuronide isomers in urine.[33,34] Most recently, this metal complexation method was applied to a large scale systematic study that allowed detailed insight into regioselectivity of UGT isozymes for five common flavonoids including hesperetin, naringenin, isorhamnetin, kaempferol, and quercetin.[54] There has also been considerable progress in modeling human UDP-glucuronsyltransferase quantitative structure/activity relationships (QSAR) and prediction of regioselectivity, as

summarized in a recent review.[55] This type of QSAR modeling has already begun to shed light on understanding the complex substrate selectivity of human UGTs.[55]

In this present study, we have expanded our investigation of the selectivity of glucuronidation of the twelve most common UGT enzymes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17) for five of the most common flavones (apigenin, baicalein, chrysin, luteolin, scutellarein) found in foods. These flavones are pervasive in common fruits, vegetables and herbs, such as celery (apigenin), scutellariae radix (baicalein), honey (chrysin), peppermint (luteolin), and scutellaria lateriflora (scutellarein). While other studies have examined UGT isozyme regioselectivities, there has been little focus on how the base structure of flavones affects this selectivity.[40,54,56] The current systematic study provides insight into the regioselectivity of UGT isozymes for flavones and also shows the unique affect that a hydroxyl group at the six position of a flavone exerts on the regioselectivity of UGT isozymes.

2.2 EXPERIMENTAL

2.2.1Materials

All UDP-glucuronosyltransferase isozymes were purchased from BD Biosciences (Woburn, MA, USA).Apigenin, baicalein, chrysin, luteolin, and scutellarein were all purchased from Indofine Chemical Co. (Hillsbrough, NJ, USA). UDP-Glucuronic acid (UDPGA) trisodium salt, 4,7-diphenyl-1,10-phenanthroline (4,7- dpphen), and cobalt(II) bromide were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade

acetonitrile, HPLC grade water, potassium phosphate, and methanol were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

2.2.2 Synthesis of flavonoid glucuronides by UGT enzymes

The procedure for the glucuronidation reactions was modified from the protocol reported in Davis et al.[34] Each enzyme was divided into 25 µL aliquots and stored at -80 °C until use. The following reaction procedure was used for each combination of UGT enzyme (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17) and flavone (apigenin, baicalein, chrysin, luteolin, and scutellarein). All volumes were delivered using appropriate micropipettes. The synthesis was carried out by adding 2 mM aqueous UDPGA (65 µL), 20 mM potassium phosphate buffer pH 7.0 (378 µL), and 10 mM methanolic solution of flavones (6.25 µL) to a microcentrifuge tube. The reaction was initiated by addition of 25 µL of a UGT enzyme (5 mg/mL). This concentration of enzyme was used based on a protocol reported by Plumb et al.[41] The mixture was incubated at 37 °C overnight. To quench the reaction, 1.5 mL of acetone was added. The final mixture was centrifuged for 10 min at 16,000 x g. The supernatant was removed and the acetone was evaporated using a Savant DNA120 SpeedVac Concentrator (Thermo Electron, Waltham, MA, USA) on low heat for 1 h 40 min. The remaining mixture was refrigerated until analysis. The activities of UGT enzymes were previously assessed in the presence of organic solvents at various concentrations, and it was reported that there were no significant changes in enzyme activities for solutions containing up to 2% methanol content.[43] Thus, the use of a minor portion of methanol (~1.25% of total volume

of solution) in the present study was not expected to be a major detriment to enzymatic activity.[42] This low concentration of methanol enhanced the solubility of the flavones and led to more accurate concentrations in solution.

2.2.3 HPLC-UV Analysis

HPLC of the flavone glucuronides was undertaken using a Shimadzu Prominence HPLC with a manual injector and 50 µL loop (Shimadzu, Columbia, MA, USA)and LCQ Duo quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) with electrospray ionization (ESI). The column was a Waters Symmetry C18 column, 2.1 mm x 50 mm, 3.5 mm particle size, with a guard column. The injection volume was 50 µL. The mobile phase was 0.33% formic acid in water (A) and 0.33% formic acid in acetonitrile (B). The gradient used began at 15% B and increased to 40% over 30 min. To evaluate the relative product distributions of different flavonoid glucuronides for each enzymatic reaction, the peak area for each resulting product was integrated based on its LC-UV chromatographic profile at 360 nm. The area of each product peak was divided by the total area of all product peaks in order to calculate the product distributions as percentages.

2.2.4 Mass Spectrometry Analysis

HPLC samples were first analyzed in the negative ESI mode in order to search for flavonoid glucuronides. The spray voltage was set at 4.5 kV, the heated capillary temperature was 200 °C, and the automatic gain control was set to 5 x 10^7 ions with a

maximum injection time of 500 ms and 5 microscans averaging. All other parameters were set to obtain optimal signal. The positive ESI mode was used for MS/MS analysis of the flavone/metal complexes. The metal complexes were formed by post-column addition of a methanolic solution of 10 mM $CoBr_2$ and 4,7-dpphen, which was infused at a rate of 20 μL/min controlled by a syringe pump. The spray voltage for the positive ion mode was set to 5 kV, and the heated capillary temperature was 200 °C. The automatic gain control for MS/MS was set to 2 x 10^7 ions with a maximum injection time of 500 ms and 5 microscan averaging, the isolation width was set to 4 Da, and a collision energy of 35% normalized collision energy was used for collision induced dissociation. For direct infusion of metal complex solutions, each flavone or flavone glucuronide was mixed in a methanolic solution with $CoBr_2$ and 4,7- dpphen in a 1:1:1 ratio. The solutions were made to have a final concentration of 10 μM. Samples were then infused at a rate of 5 μL/min via a syringe pump. The rest of the MS parameters were kept the same as that used for the samples analyzed by LC-MS.

2.3 RESULTS

After reaction of the flavones in the presence of the glucuronysyltransferases, identification of the products as glucuronides is straightforward by LCMS due to the characteristic mass shift (+176 Da) due to attachment of the glucuronyl moiety. However, the flavones with multiple hydroxyl groups produced one or more glucuronides upon incubation with each glucuronosyltransferase, yielding isobaric products. The MS/MS spectra of the resulting isobaric flavone glucuronides are too similar to allow their

differentiation. An alternative approach utilizing the MS/MS spectra of the metal complexes [Co(II) (FG–H) (4,7-dpphen)$_2$]$^+$ (where FG represents a flavone glucuronide) in conjunction with the absolute or relative chromatographic retention times allowed differentiation and assignment of the various flavone glucuronides, including isomers. A postcolumn complexation method was used to generate the [Co(II) (FG–H) (4,7-dpphen)$_2$]$^+$ upon elution of each glucuronide, and these complexes gave distinctive, diagnostic fragmentation patterns upon CID, thus giving more confident identification than obtained for deprotonated or protonated flavone glucuronides.[30,34,54] For example, metal complexes incorporating 7-O-glucuronides exhibit losses of the auxiliary ligand (-Aux), the glucuronic acid moiety (-GlcA), or both (-(GlcA + Aux)), upon CID. The 7-O-glucuronides also demonstrate the loss of the flavone aglycon (-Agl) to a lesser extent. In contrast, the metal complexes of 5-O-glucuronides generally undergo a prominent loss of the glucuronic acid moiety along with the auxiliary ligand (-(GlcA + Aux)); additionally, the 5-O product characteristically elutes before the 7-O product.[34] The 6-O-glucuronides do not dissociate by elimination of the aglcyon moiety, thus allowing them to be differentiated from the 7-O-glucuronides. B-ring-glucuronides dissociate via the loss of the auxiliary ligand (-Aux) as well as the combined losses of both the auxiliary ligand and glucuronide moiety (-(GlcA + Aux)).[30] 4'-O-glucuronides elute prior to 3'-O glucuronides and after the corresponding 7-O-glucuronides. As summarized briefly here, these characteristic elution orders and fragmentation patterns allow facile differentiation of isomeric flavonoid glucuronides.

With respect to the implementation of this approach, the flavone glucuronides were derived from the supernatants obtained after centrifugation of the enzymatic reaction incubates. The glucuronides were separated and then ionized by either negative ESI or via post-column metal complexation prior to introduction into the ion trap mass spectrometer. To pinpoint the elution of the flavone products of interest, specific m/z values corresponding to each unmodified flavone and its monoglucuronidated (aglycon + 176), and diglucuronidated (aglycon + 176 + 176) products were searched in the total ion chromatograms. In the present study, no diglucuronidated products were found. Collision induced dissociation (CID) of the positively charged flavone glucuronide/metal complexes, not the deprotonated flavone glucuronides, yielded the most distinctive fragmentation patterns that confirmed the identity of each species. Examples of the MS/MS spectra for some of the metal complexes are shown in Figure 2.2 for the luteolin glucuronides produced from UGT1A1 and in Figure 2.3a and 2.3b for the baicalein glucuronides produced from UGT1A1. All products identified based on the unique MS/MS patterns of the metal complexes are summarized in Table 2.1 along with the quantitative distribution of products obtained by integration of the chromatographic peak areas of each product and unmodified flavone.

The glucuronide products of four additional flavones, including three with just a single hydroxyl group at the 5, 6, or 7 position, as well as one flavone with hydroxyl groups at the 6 and 7 positions, were also evaluated in order to provide additional confirmatory evidence about the relative retention times of flavones modified at the 5, 6 or 7 position. The additional flavones are listed in Figure 2.1, and the MS/MS patterns of

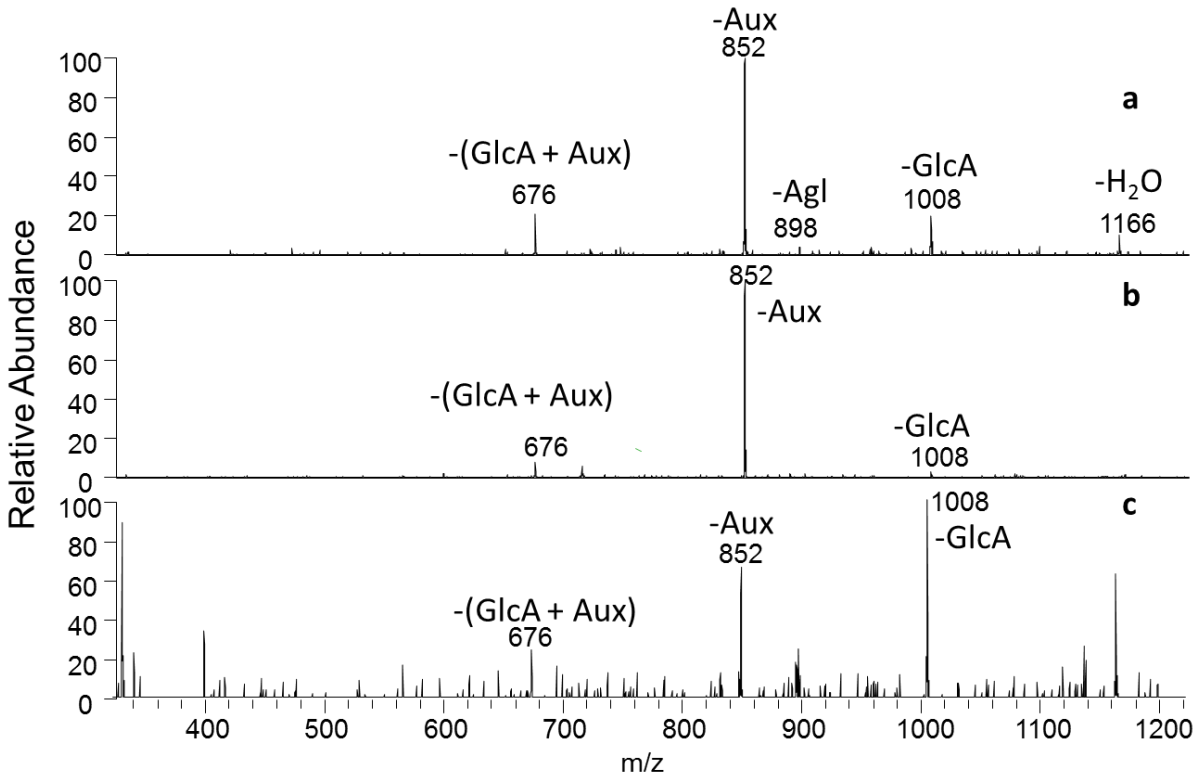


Figure 2.2: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A1/Luteolin products: a) 7-O glucuronide, m/z 1184 b) 3'-O glucuronide, m/z 1184 c) 4'O- glucuronide, m/z 1184. –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon); -(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand)

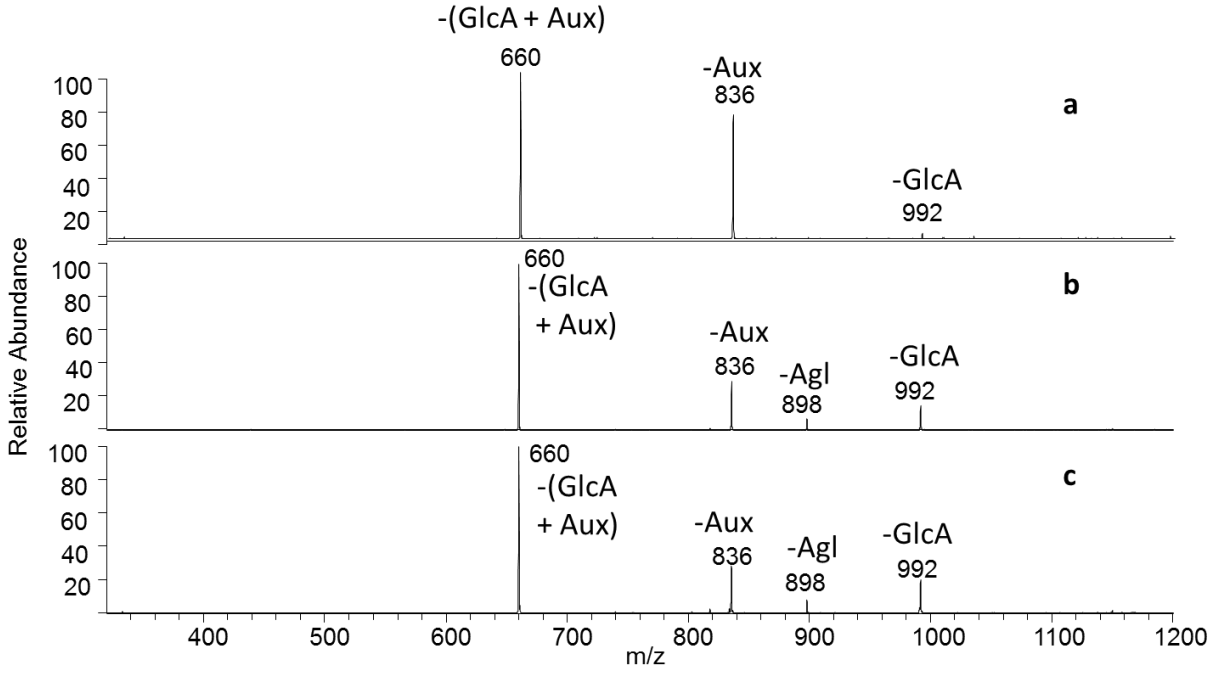


Figure 2.3: CID mass spectra of [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ for all UGT1A8/Baicalein products compared to CID of baicalein 7-O glucuronide standard : a) 6-O-glucuronide, m/z 1168 b) 7-O-glucuronide, m/z 1168 c) 7-O glucuronide standard, m/z 1168. –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon); -(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand)

Table 2.1: Glucuronide product distributions

Chrysin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
Chrysin	35	Trace	100	30	100	40	20	80	100	65	100	100
5-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
7-O-Glucuronide	65	100	Trace	70	Trace	60	80	20	-	35	Trace	Trace
Apigenin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
Apigenin	35	70	100	80	100	65	10	75	100	95	100	100
5-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
7-O-Glucuronide	65	30	-	20	-	35	90	25	-	5	-	-
4'-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
Luteolin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
Luteolin	40	80	100	100	70	55	5	85	100	70	100	100
5-O-Glucuronide	-		-	-	-	-	-	-	-	-	-	-
7-O-Glucuronide	10	10	-	-	10	10	40	5	-	5	-	-
3'-O-Glucuronide	20	5	-	-	15	20	40	10	-	25	-	-
4'-O-Glucuronide	30	5	-	-	5	15	15	Trace	-	Trace	-	-
Baicalein	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
Baicalein	5	5	100	Trace	100	Trace	Trace	Trace	100	100	100	100
5-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
6-O-Glucuronide	-	-	-	-	-	70	5	20	-	-	-	-
7-O-Glucuronide	95	95	-	100	-	30	95	80	-	-	-	-
Scutellarein	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
Scutellarein	30	Trace	100	5	100	Trace	Trace	5	100	100	100	100
5-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-
6-O-Glucuronide	-	-	-	-	-	50	25	20	-	-	-	-
7-O-Glucuronide	70	100	-	95	-	50	75	75	-	-	-	-
4'-O-Glucuronide	-	-	-	-	-	-	-	-	-	-	-	-

All values are percentages of total product distribution. A dash is used to indicate the absence of a product. The average standard deviation was ± 6%. All values rounded to the nearest 5%.

the [Co(II) (FG–H) (4,7-dpphen)$_2$]$^+$ complexes of the corresponding glucuronides are shown in Figures 2.4 and 2.5. Interestingly, the fragmentation patterns of the glucuronide products generated from the simple 6-hydroxy and 7-hydroxy flavones do not display the identical multiple pathways noted for the flavones that possess multiple hydroxyl groups. For example, the only pathway for the glucuronidated 6-hydroxyflavone is the loss of the auxiliary ligand (-Aux) (Figure 2.4A), and the most dominant fragmentation pathway for the glucuronidated 7-hydroxyflavone is the loss of the glucuronic acid group (-GlcA) (Figure 2.4B). This notable simplification of the fragmentation patterns is not surprising. The streamlined mono-hydroxyl flavones do not have multiple metal coordination sites like the other multi-hydroxyl flavones, and thus the array of possible metal-chelation structures that lead to diagnostic fragment ions is correspondingly reduced, thus yielding simpler MS/MS patterns. This point is clearly demonstrated by comparison of the MS/MS patterns of the glucuronide of 6-hydroxyflavone Fig 2.4A), 7-hydroxyflavone (Fig 2.4B), and the two glucuronides of 6,7-dihydroxyflavone (Fig 2.5). Whereas the fragmentation patterns in Fig 2.4 show only a single product, the ones in Fig 2.5 exhibit a richer series of diagnostic fragment ions (i.e., *loss of glucuronic acid moiety, loss of aglyone, loss of auxiliary ligand, loss of both glucuronic acid and auxiliary ligand*) that allow ready differentiation of the 6-O and 7-O glucuronides. In short, the relative retention times but not the MS/MS patterns of the simplest mono-hydroxyl flavones are useful for supporting the assignment of glucuronide products of the multi-hydroxylated flavones. With respect to the retention times, the 7-O-glucuronides consistently elute sooner than the 6-O-glucuronides, thus providing important confirmatory evidence.

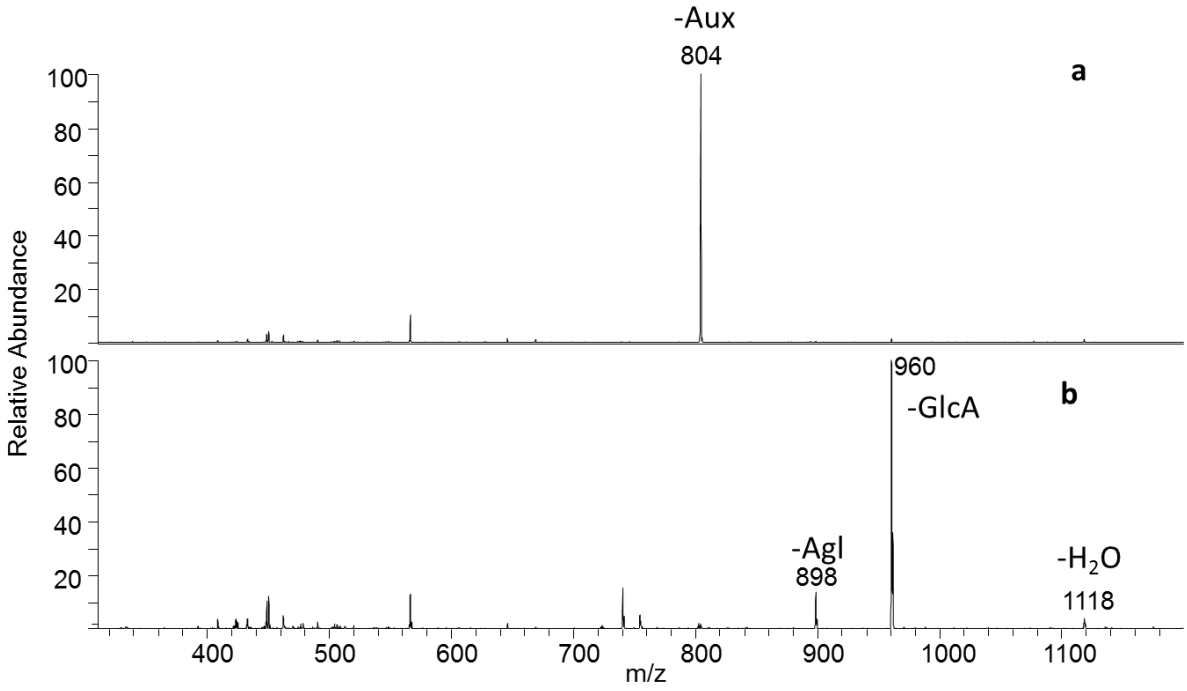


Figure 2.4: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for a) UGT1A8/ 6-hydroxyflavone product 6-O glucuronide, m/z 1136 b) UGT1A3/ 7-hydroxyflavone product 7-O glucuronide, m/z 1136. –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon)

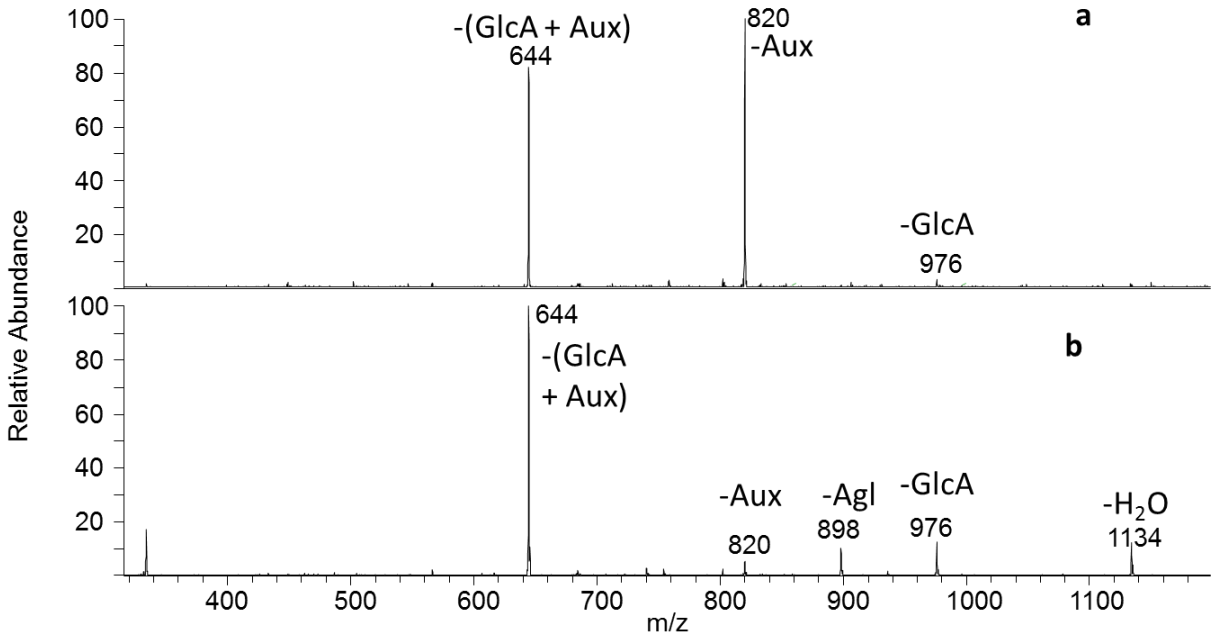


Figure 2.5: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A9/ 6,7-dihydroxyflavone products a) 6-O glucuronide, m/z 1152 b) 7-O-glucuronide, m/z 1152. –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon); -(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand)

The 5-hydroxyflavone formed one detectable glucuronide product upon incubation with the UGT isozymes, as indicated based on observation of a presumed deprotonated glucuronide upon LC/MS. However, this 5-O glucuronide did not form stable metal complexes, thus preventing inclusion of its MS/MS pattern in Figure 2.4. Glucuronidation at the 5-O position inhibits metal coordination between that position and the nearby keto group, thus explaining the lack of metal complexes. Glucuronidation at the 5-O position was not found for any of the multi-hydroxylated flavones described in this study, suggesting that the 5-O position is the least favorable when other sites are available.

2.3.1 Identification of Flavone Glucuronides

Two flavones, chrysin and apigenin, produced at most one characteristic monoglucuronide when reacted in the presence of each UGT isozyme. The reactions with apigenin resulted in the same monoglucuronide product for UGT1A1, 1A3, 1A6, 1A8, 1A9, 1A10, and UG2B7 but no products for UGT1A4, 1A7, 2B4, 2B15, 2B17. The sole product exhibited losses of GlcA, Agl, and Aux, a pattern which is characteristic of 7-O-glucuronide products (see CID mass spectra in Fig. 2.6). Chrysin, on the other hand, formed a single product for all glucuronosyltransferases except for UGT2B4 which resulted in no products. The single monoglucuronidated product from chrysin dissociated by pathways characteristic of a 7-O-glucuronide (losses of GlcA, Agl, and Aux). The corresponding MS/MS data for the chrysin 7-O- glucuronide is shown in Figure 2.7.

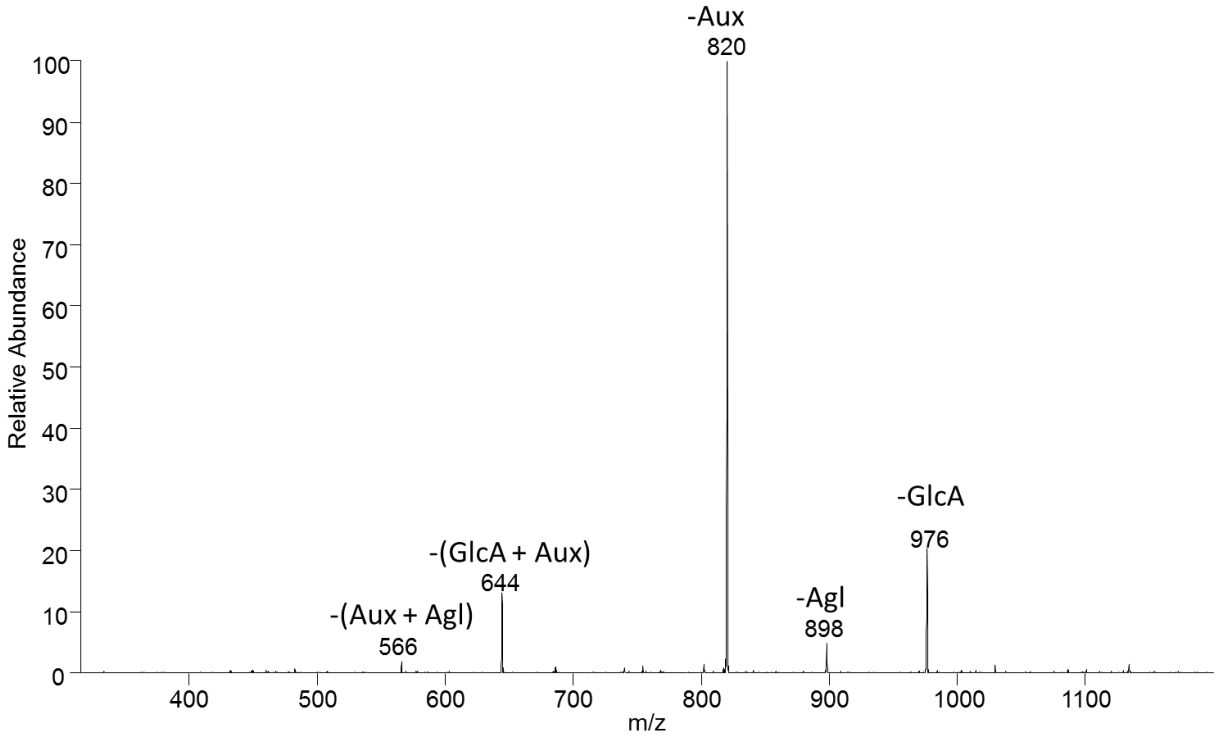


Figure 2.6: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for the UGT1A1/Chrysin product: 7-O glucuronide, m/z 1152 . –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon); -(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand)

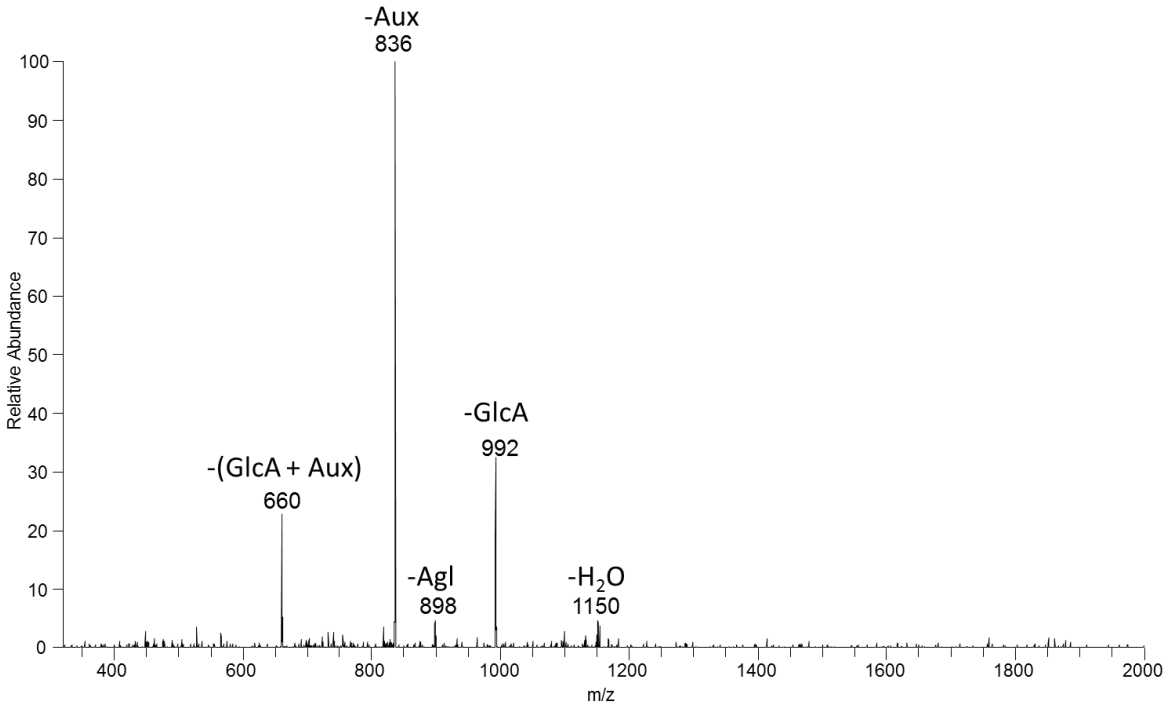


Figure 2.7: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for the UGT1A1/Apigenin product: 7-O glucuronide, m/z 1168 . –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon); -(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand)

Luteolin, with three hydroxyl groups, generated three different products upon reaction in the presence of UGT1A1, 1A3, 1A7, 1A8, 1A9, 1A10, and 2B7. The first eluting product dissociated by losses of GlcA, Agl, and Aux, all which are consistent with a 7-O-glucuronide product. The next two products each displayed losses of Aux, (Aux + GlcA), and GlcA upon CID. Since 4'-O-glucuronide products typically elute before 3'-O-glucuronide products, the two species are identified as the 4'-O and 3'-O products, respectively. (see Figure 2.2).

A single product was observed for baicalein when modified in the presence of UGT1A1, 1A3, and 1A6 and two glucuronides were produced upon reactions with UGT1A8, 1A9, 1A10. The product formed from the 1A1, 1A3, and 1A6 reactions showed characteristic losses of Aux, GlcA, and (GlcA + Aux). These are also the same fragments observed for the first eluting product of the 1A8, 1A9, and 1A10 reactions. The (GlcA + Aux) ion is the dominant fragment ion in the spectra, in addition to less abundant ions representing losses of Aux and GlcA. This MS/MS pattern is the same fragmentation pattern seen for the 7-O glucuronide of the 6,7-dihydroxyflavone discussed earlier. The second product from the reactions of baicalein with UGT1A8, 1A9, and 1A10 showed prominent fragments attributed to the loss of Aux or the loss of (Aux + GlcA) which matches the dissociation pattern of the 6-O glucuronide product from the 6,7-dihydroxyflavone reaction. The structural assignment of these two bacalein glucuronides was confirmed by comparing these fragmentation patterns to that obtained for a commercially available reference compound, baicalein 7-O-glucuronide. Upon CID, the latter exhibited the loss of Aux or GlcA as well as a dominant fragment

corresponding to the loss of (Aux + GlcA) (Figure 2.3c). Based on this evidence as well as the retention time of baicalein 7-O-glucuronide (see Figure 2.8a) relative to the retention times of the two baicalein-glucuronide products (See Figure 2.8b), it is clear that the single product formed by bacalein upon reaction with UGT1A1, 1A3, and 1A6 and the first eluting product upon reaction of bacalein with UGT 1A8, 1A9, and 1A10 corresponds to glucuronidation of the 7-O position. This also allows the confident identification of the second product as a 6-O-glucuronide based on its greater retention time relative to the 7-O glucuronide (as described earlier for the model flavones).

This LC-MS/MS strategy also allows confident assignment of the products of the scutellarein glucuronidation reactions. Similar to bacalein, scutellarein also formed a single glucuronide when incubated with UGT1A1, 1A3, 1A6 and two products when incubated with UGT1A8, 1A9, and 1A10. Based on the MS/MS patterns and relative retention times, the single product from the UGT1A1, 1A3, and 1A6 reactions and the first eluting species from reaction of UGT1A8, 1A9, and 1A10 is attributed to a 7-O-glucuronide product. The second eluting product of the UGT1A8,1A9, and UGT1A10 reactions shows losses of both Aux and (Aux + GlcA), analogous to the pattern seen for the 6-O modification of the baicalein, so this product can be identified as a modification of the 6-O position of scutellarein (see CID mass spectra in Fig. 2.9).

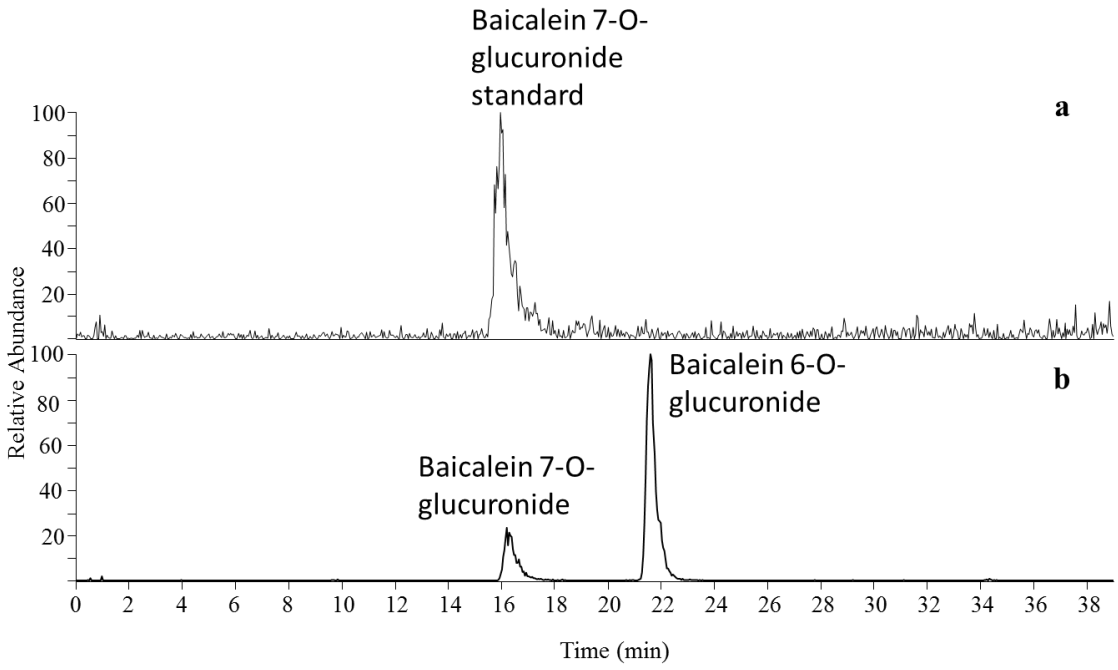


Figure 2.8: Selective ion chomatogram (m/z 445) for a) Baicalein 7-O-glucuronide b) UGT1A8/Baicalein products.

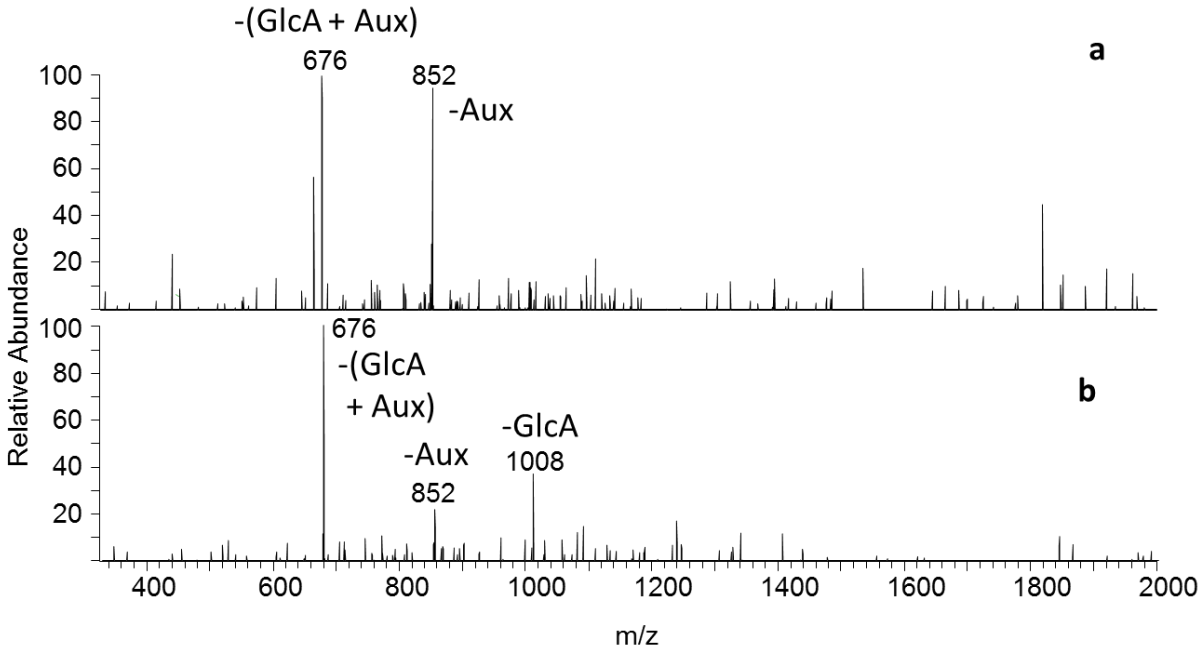


Figure 2.9: CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A8/ Scutellarein products a) 6-O glucuronide, m/z 1184 b) 7-O- glucuronide, m/z 1184. –Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); -Agl (loss of flavonoid aglycon); -(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand)

2.3.2 Selectivity Trends

7-Hydroxyflavone, chrysin, apigenin and luteolin afford an interesting series for comparison of how additional hydroxyl groups affect glucuronidation site selectivity because each of these flavones has an increasing number of hydroxyl groups, starting with the standard 7-OH, then adding the 5-OH, then adding the 4'-OH, then the 3'-OH for each flavone in the series. Singh *et al.* investigated the selectivity of UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7 with chrysin and apigenin by using a UV shift method.[40] Singh *et al.* reported that apigenin and chrysin were glucuronidated solely at the 7-O position, along with minor modification at the 5-O position of apigenin for UGT1A6 and 1A10. Our results agree with Singh's findings with the exception that we observed no glucuronidation reactions with UGT1A7 nor was glucuronidation at the 5-O position observed for apigenin. Comparing the results obtained for chrysin and apigenin, it appears that a hydroxyl group at the 4' position of a flavone has little to no effect on the selectivity the UGT isozymes as there is virtually no difference in the product distributions for chrysin and apigenin for all the UGT isozymes. Luteolin provides a unique opportunity to observe how UGT selectivity changes upon addition of another hydroxyl group at the 3' position. Interestingly, the addition of the second hydroxyl to the B-ring of flavone results in modification of both B-ring sites for all active UGT isoforms.

Baicalein and scutellarein each have three potential glucuronidation sites on the A ring (5-OH, 6-OH, and 7-OH) and are also the only two flavones that have the 6-OH

group. Chen *et al.* reported that no glucuronidation of baicalein occurred for reactions with UGT1A3 and UGT1A9.[35] In the current study we found baicalein does in fact form abundant monoglucuronide products in the presence of all UGT1A isozymes except for UGT1A4, 1A7 and exhibits no reaction with any of the UGT2B isozymes. Scutellarein showed similar activity as baicalein with all UGT isozymes. The site selectivity for bacalein and scutellarein are similar, suggesting that the extra hydroxyl group at the 4' position for scutellarein is a non-reactive site. For baicalein and scutellarein, incubation with UGT 1A8, 1A9, and 1A10 result in the formation of 6-O-glucuronides.

All flavones show limited reactivity with the UGT2B isozymes. This same trend was noticed previously for glucuronidation of flavonols in our earlier study in which it was hypothesized that the planar nature of these compounds restricted their modification by the UGT2B isozymes, in contrast to the ample glucuronidation observed for flavanones (a class of flavonoids that lack the 2-3 double bond, thus allowing greater conformation flexibility of the C ring).[54]

2.4 CONCLUSIONS

This study provides insight into the regioselectivity of twelve UGT isozymes for five naturally occurring flavones and demonstrates the differentiation of glucuronide isomers that is essential for bioavailability and biotransformation studies. The formation and CID analysis of metal complexes of the type ([Co(II) (flavone glucuronide - H) (4,7-dpphen)$_2$]$^+$) via post-column addition of a metal/ligand solution was a key analytical strategy that allowed differentiation of isobaric products, most of which give identical

MS/MS fragmentation patterns for the conventional deprotonated species. UGT isozyme selectivity is affected by the presence of a hydroxyl group at the 3' position, as luteolin is the only flavone that exhibited glucuronidation of the B-ring. For baicalein and scutellarein, three of the UGT1A isozymes (1A8, 1A9, and 1A10) resulted in the formation of 6-O glucuronides, enabling the fragmentation rules for the metal complexation/MS/MS strategy to be expanded. Consistent with our previous results for flavonols, the planar structure of the flavones prohibits their glucuronidation by the UGT2B isozymes.

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